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Melipona beecheii (Hymenoptera, Apidae) foragers deposit a chemical mark on food to attract conspecifics

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Abstract

Stingless bees have a sophisticated system of chemical communication that helps conspecifics find food sources. In this study, we investigated whether *Melipona beecheii* foragers deposit a chemical mark on food to recruit conspecifics. Our results showed that foragers preferred to visit the feeders visited previously by conspecifics over clean feeders. We also found that foragers preferred visiting feeders baited with labial gland extracts over those baited with mandibular extracts or hexane. Labial gland extracts elicited higher forager antennal responses compared with those evoked by the mandibular gland extracts or hexane. Labial gland extracts and extracts from feeders visited by foragers contain a mixture of unsaturated hydrocarbons, followed by straight chain hydrocarbons and small quantities of esters. The main component is a mixture of alkene isomers C27:1.

Keywords

CG-MS, cuticular hydrocarbons, labial gland, stingless bees

Introduction

Eusocial bees, such as *Apis mellifera* and stingless bees, use communication mechanisms such as vision, smell for food search and mechanical signals (Dyer 2002; Nieh 2004; Barth et al. 2008; Dyer et al. 2016). These mechanisms are species-specific and make social bees highly efficient in locating and exploiting food resources. For example, several bee species can recognize whether the flower resources have been exhausted and if so, reduce their searching time (Stout and Goulson 2001).

A typical method of communication happens when stingless bee foraging workers arrive at their nest with food; then they release chemical signals to stimulate other workers to go out to the field in search of food sources (Nieh 2004). *Trigona* and *Scaptotrigona* species display another form of communication. Here, foragers use chemicals from the labial glands as a tracking pheromone to mark the paths to food sources (Jarau et al. 2004a, 2006; Schorkopf et al. 2007; Hemmeter 2008; Stangler et al. 2009; Jarau et al. 2011). When marking the paths, the bee extends its proboscis and rubs it against the substrate to deposit the chemical trail produced by the labial glands (Jarau et al. 2004a). In contrast, *Melipona* foragers do not lay out chemical paths to recruit conspecifics to the food site (Hrncir et al. 2004); they deposit chemical marks on the food source to attract conspecifics (Jarau et al. 2004b). However, the source of these marks is unclear and is occasionally controversial. For instance, *Melipona favosa* foragers deposit anal excretions on food sources (Aguilar and Sommeijer 2001), while *Melipona panamica* and *Melipona scutellaris* foragers deposit chemical marks that influence the conspecifics orientation toward a food source. Notwithstanding the precise source is known, the chemical identity of these signals has been reported only in a few species (Nieh 1998; Hrncir et al. 2004; Jarau et al. 2004b; Roselino et al. 2016), such as, *Melipona solani* which foragers leave a mixture of hydrocarbons from their cuticle and methyl oleate from the labial gland as an odor mark on the food source (Alavez-Rosas et al. 2017). However, it is not known if this communication mechanism occurs in other *Melipona* species. Consequently, we investigated whether *Melipona beecheii* foragers deposit marks on food to attract their conspecifics. Our hypothesis is that *M. beecheii* foragers deposit chemical marks on food to attract their conspecifics. *M. beecheii* is one of the stingless bees species with high ecological, economical, and cultural importance in Mexico. Particularly, *M. beecheii* stingless bees are highly valuable in meliponiculture and crop pollination in Mexico. In spite of this, little is known of its chemical ecology (Ayala et al. 2013).

Materials and methods

Biological material

We used five *M. beecheii* (Hymenoptera, Apidae, Meliponini) colonies obtained from a meliponary in Tuxtla Chico (14°56'25"N, 92°10'08"W), Chiapas, Mexico. The experiments were conducted from April to October 2021 at El Colegio de la Frontera

Sur gardens, Tapachula (14°54'39.86"N, 92°15'51.55"W), Chiapas, Mexico. Annual rainfall in this region is approximately 3843 mm, with September as the wettest month and February as the driest month. The temperature normally ranges between 29 °C and 35 °C. The colonies were free of fungi and parasites.

Training

The forager bees were trained to collect a solution of 3 M sucrose *ad libitum* from an artificial feeder, consisting of a Petri dish (100 × 10 mm) containing a small cotton ball drenched in the sucrose solution in the center. The feeder was placed 5 m from the beehives. The training was conducted between 08:00 and 13:00 h.

Collection of chemical marks

We extracted the compounds deposited by *M. beecheii* foragers on a glass feeder (100 × 10 mm) (Hrncir et al. 2004) by washing the feeders with 4 mL hexane (HPLC grade, Aldrich, Toluca, Mexico). The amount of compounds left by 40 or 50 foragers on the food source was considered as a biological active mark. The hexane extracts were concentrated to 50 µL using a gentle flow of N_2 and stored at -20 °C until their analysis. Five different extracts were obtained.

Gland extracts

To prepare the gland extracts, we captured foragers bees that arrived at the feeder during training. Bees were frozen at -20 °C before dissection and analysis. The glands of the foragers were carefully dissected in distilled water with two pairs of fine tweezers under a stereoscopic microscope. The gland extracts were prepared by carefully dissecting the labial and mandibular glands of 10 foragers in 1000 µL of solution. So, 100 µL of the prepared solution is the amount corresponding to 1 labial gland equivalent (LGE), 50 µL of solution corresponds to 0.5 LGE and 10 µL to 0.1 LGE; and 100 µL of the prepared solution is the amount corresponding to 1 mandibular gland equivalent (MGE), 50 µL of solution corresponds to 0.5 MGE and 10 µL to 0.1 MGE. Five gland extracts were prepared.

Behavioral bioassays

In a first experiment, we evaluated whether the forager bees leave odor marks on the feeders in a two-choice tests. We offered to foragers two feeders: one with chemical marks (previously visited by foragers) and the other clean (not visited by foragers). The feeders were placed at the site where the bees were trained. Feeders were placed 30 cm from each other. We placed a few drops of the 3M sucrose solution in the entrance to stimulate the visits. We recorded the number of bees that visited the marked feeder and the clean feeder. A visit was counted when the bee landed and extended its proboscis to feed. All bees were marked with a fine brush with acrylic paint not toxic and captured

on their first visit to avoid counting the same bee more than once (Alavez-Rosas et al. 2017). To prevent the phenomenon of social facilitation (influence in the bee election due to the presence of a conspecific at the feeding site), care was taken not to count the bees that visited the feeders while other bees were there. The position of the feeders was interchanged every 5 min to prevent position bias. At the end of the trial, the bees were freed near the colony. All trials were conducted between 08:00 and 13:00 h. In a total, 10 replications were performed.

In a second experiment, we assessed the effect of the labial gland extracts on food searching in two choice tests. Here, using pieces of 1cm² filter paper placed on feeders, one feeder was sprinkled with 10 µL of labial gland extract at the beginning of the experiment and the other feeder was sprinkled with 10 µL of hexane as a control. The gland extracts were evaluated at 0.1, 0.5, and 1 LGE. In total, 10 replications were performed for each gland equivalent extract.

In a third experiment, we evaluated foragers preference for labial gland extract, mandibular gland extract, or solvent in three-choice tests. Three feeders were placed in the training site: control (hexane), labial gland extract, and mandibular gland extract. The feeders were placed 20 cm from each other. Care was taken not to count bees that visited the feeder while other bees were there to avoid the phenomenon of social facilitation. The position of the feeders was interchanged every 5 min to avoid position bias. The gland extracts were evaluated at 0.1, 0.5, and 1 LGE or MGE. In total, 10 replications were performed for each gland equivalent extract.

Electroantennography (EAG)

We collected forager bees from three established colonies, bees were frozen for one minute to numb them before dissection, subsequently their antennae were carefully removed. The base of the antenna was inserted into the tip of the glass capillary logging electrode. The signals generated by the antenna passed through a high impedance amplifier (NL 1200; Syntech, GmbH) and visualized with the software Syntech to process the EAG signals. We used a stimulus Flow controller (CS-05, Syntech) to generate stimuli at intervals of one minute. A constant current of pure humidified air (0.7 L min^{-1}) was directed toward the antenna (Malo et al. 2004).

The experimental procedure consisted of depositing the treatment (1 LGE extract, 1 MGE extracts or solvent, in this order) onto 1.5×1.5 mm pieces of filter paper (Whatman no. 1, Whatman, Maidstone, England) exposed to air for 20 s to allow the solvent to evaporate, inserted into a glass Pasteur pipette, and left for 40 s before applying. The application of the stimulus consisted of inserting the tip of the pipette that contained the piece of filter paper in a hole at the end of the glass tube through which the current of air blew continuously on the antenna. The waiting time between one stimulus and the next was one minute. The treatment was carried from the filter paper to the antenna on the controlled air current (0.5 L/min). The duration of the stimulus was 1 s. The continuous flow of pure air was maintained through the tube to assure that the odors were removed immediately. We used one antenna of the bee per treatment, and at least thirty five bees were used.

Chemical analysis

Extracts were analyzed in a gas chromatograph (Shimadzu GC-2010 Plus) coupled with a quadrupole mass spectrometer (Shimadzu, TQ8040), using a capillary column of non-polar silica SPB-1 (30 m long × 0.25 mm interior diameter) (Supelco, Toluca, Mexico). The initial temperature was 50 °C (held for 2 min), increased 15 °C/min up to 280 °C (held 10 min). Helium was the carrier gas and the injector temperature was 250 °C. Ionization was achieved by electron impact at 70 eV. The compounds were identified by comparison with those registered in the NIST 2014 library (software GCMS-solution), the retention index, the mass spectra, and retention times of available synthetic standards. The relative percentage of the components was calculated from the sum of the recorded peaks.

Bioassay with synthetic compounds

We evaluated the mixture of synthetic compounds with some compounds identified in the labial gland secretion. The synthetic blend evaluated was prepared in accord with the natural proportions of the *M. beecheii* labial gland using hexane as solvent. The compounds evaluated were heneicosane (100 ng), tricosane (100 ng), and pentacosane (25 ng). These compounds were chosen as they were available in supplies. Unfortunately, C27:1 isomers the main components in the labial gland secretion were not commercially available. We recorded the number of bees that visited a feeder baited with the synthetic blend, or a feeder with solvent. The feeders were placed where the bees were trained to visit. The distance between the feeders was 30 cm. The position of the feeders was changed every 5 min to avoid position bias. In total, 10 replications were performed in this experiment.

Statistical analysis

All data were analyzed using R software (R Core Team 2020). The EAG data were natural logarithm transformed and analyzed by a one-way analysis of variance (ANOVA), followed by a Tukey test. Behavioral data were transformed to satisfy the assumptions of normality and homoscedasticity and analyzed by generalized linear model (GLM) to the Poisson or negative binomial models.

Results

Behavioral bioassays

M. beecheii foragers preferred the feeders visited previously by their conspecifics over the clean feeder ($\chi2 = 56.783$, df = 1, p < 0.001) (Fig. 1).

In the two-choice bioassays, foragers preferred to visit the feeders marked with extracts of 0.5 LGE ($\chi2 = 134.38$, df = 1, p < 0.001) and 1 LGE ($\chi2 = 71.676$, df = 1,
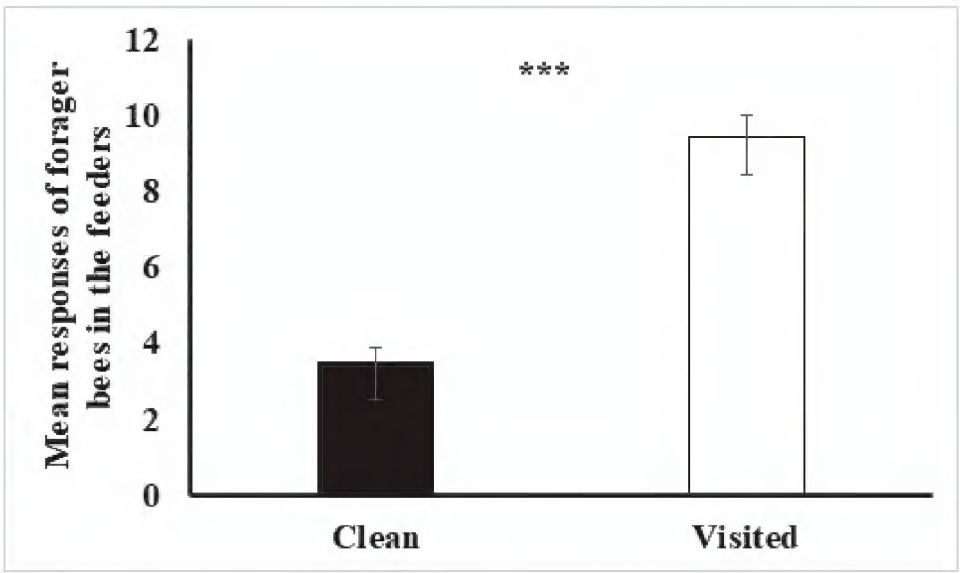
Figure 1. Mean (± SEM) responses of *M. beecheii* foragers to marked and clean feeders (*** P< 0.001).
Ten replications were performed.
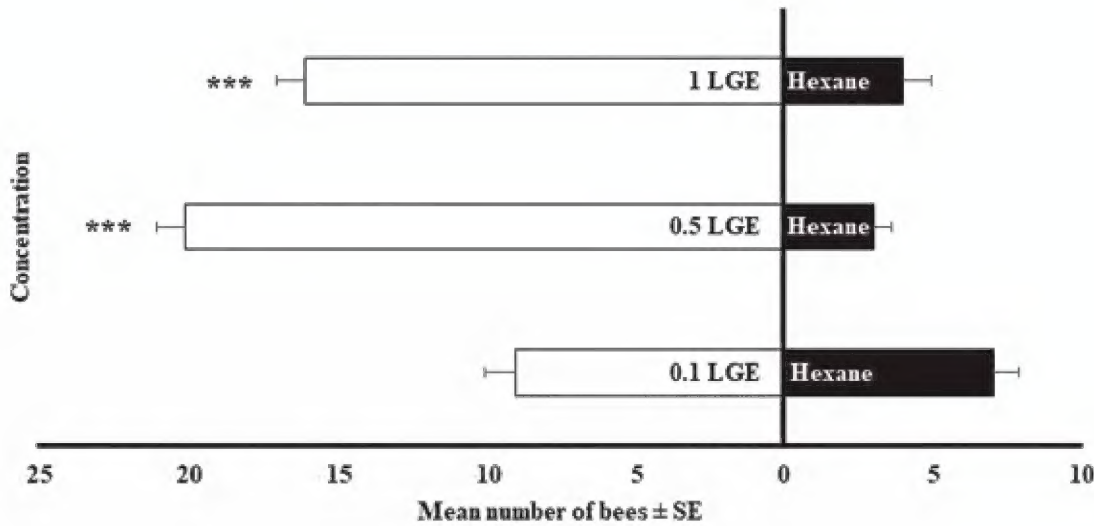
Figure 2. Mean (± SEM) responses of foraging *M. beecheii* worker bees to labial gland extract from conspecific worker bees at different concentrations (LGE=labial gland equivalent, *** P< 0.001, ** P<0.01, * P< 0.05). Ten replications were carried out for each gland equivalent extract.
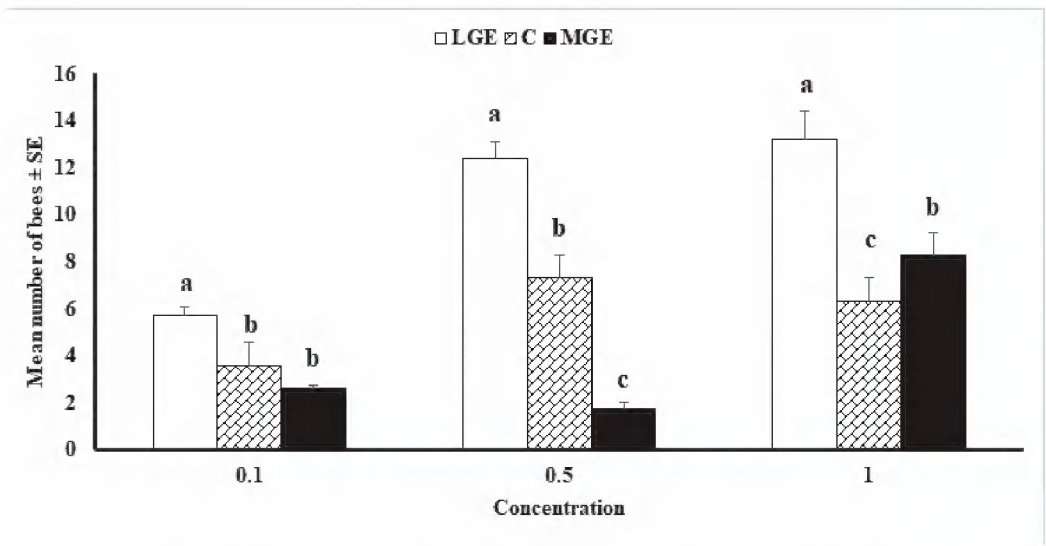
Figure 3. Mean (± SEM) responses of *M. beecheii* foragers to labial gland and mandibular gland extracts at different concentrations. Different small letters (*P*< 0.05) indicate significant differences between treatments. Ten replications were performed for each gland equivalent extract. LGE=labial gland equivalent, MGE=mandibular gland equivalent, C=control.

p < 0.001) over those treated with hexane. However, they did not show a preference for the 0.1 LGE or the control ($\chi2$ = 0.53236, df = 1, p = 0.4656) (Fig. 2).

In the three-choice bioassays, more foragers preferred visiting feeders with labial gland extracts (0.1 LGE: $\chi2$= 13.335, df = 2, p<0.01; 0.5 LGE: $\chi2$= 81.747, df = 2, p < 0.001; 1 LGE: $\chi2$ = 23.929, df = 2, p < 0.001) over other feeders with mandibular extracts or the control (Fig. 3).

Electroantennography

Analysis of EAG data revealed significant differences in the antennal response of forager bees to the different extracts evaluated and to the solvent (control) (F = 13.24, df = 2, P > 0.001). The antennal responses of the foragers was greater with 1 eq/µL of labial gland extract than with the mandibular gland extract or with the control (Fig. 4).

Chemical analysis

Chemical analysis showed that the labial gland extracts and extracts from feeders visited by foragers contain a mixture of unsaturated hydrocarbons, followed by straight chain hydrocarbons and small quantities of esters. The main components are a mixture of alkene isomers C27:1 (Table 1).

The chromatographic profile of the hexane feeder wash was similar to the labial gland extract profile, but different from that of the mandibular gland extract (Fig. 5).

Bioassay with synthetic compounds

Foragers did not show a preference for the feeders treated with a three-component blend and those treated with hexane.
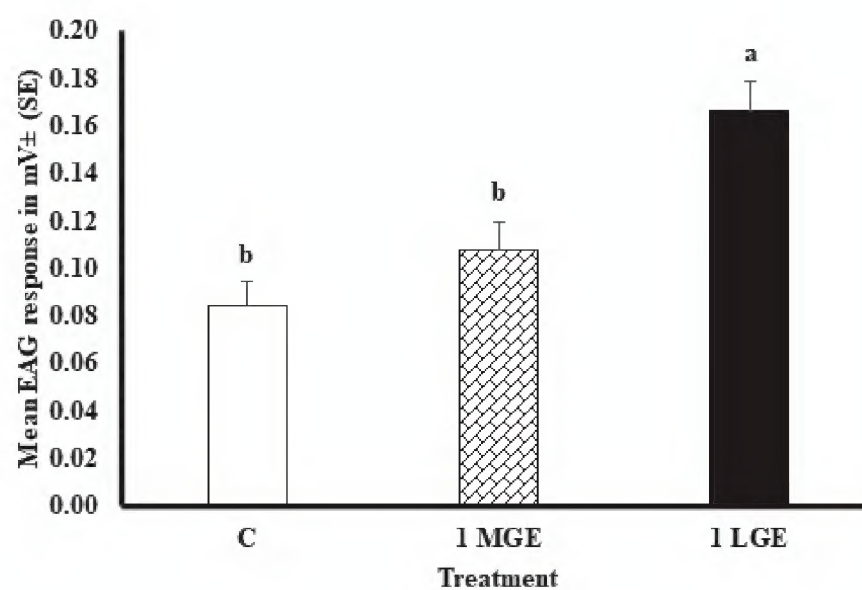

Figure 4. Electroantennographic (EAG) response in mV of *M. beecheii* forager bee antennae to labial gland extract, mandibular gland extract and the control (C). Different small letters (*P*<0.05) indicate significant differences between treatments. N=35. LGE=labial gland equivalent, MGE=mandibular gland equivalent, C=control.

Table 1. Average value (%) ± standard error of the proportion of the compounds found in the labial glands of *M. beecheii* forager bees. N=Five gland extracts.

Peak	RT	RI	Compound	Proportion in labial gland	Proportion on feeder
1	13.53	1778.50	Alcohol	2.07±0.40	ND
2	15.57	2100.00	Heneicosane (C21)*	0.26±0.15	ND
3	15.85	2124.75	Methyl stearate *	0.4±0.07	ND
4	15.97	2143.48	Geranyl palmitate **	2.79±0.48	ND
5	16.11	2166.93	2,3-Dihydro farnesyl hexanoate**	1.89±0.31	ND
6	16.25	2189.90	Unknown 1	1.28±0.22	ND
7	16.33	2202.45	Farnesyl butanoate**	1.88±0.30	ND
8	16.74	2274.56	Alkene C23:1 (1)	0.63±0.26	0.81±0.47
9	16.78	2281.22	Alkene C23:1 (2)	0.61±0.18	0.12±0.07
10	16.88	2300.00	Tricosane (C23)*	0.26±0.07	0.30±0.18
11	17.90	2474.00	Alkene C25:1 (1)	7.09±0.60	0.74±0.42
12	17.94	2481.16	Alkene C25:1 (2)	2.17±0.20	0.19±0.11
13	18.04	2500.00	Pentacosane (C25)*	0.6±0.05	0.24±0.14
14	18.20	2570.25	Unknown 2	3.37±0.75	ND
15	19.29	2672.08	Alkene C27:1 (1)	8.33±0.87	1.21±0.70
16	19.33	2684.35	Alkene C27:1 (2)	8.05±0.81	1.38±0.80
17	19.39	2698.00	Alkene C27:1 (3)	11.2±1.10	1.37±0.79
18	19.51	2700.00	Heptacosane (C27)*	2.89±0.27	5.52±3.19
19	21.07	2853.12	Alkene C29:1 (1)	3.95±0.34	ND
20	21.14	2875.78	Alkene C29:1 (2)	1.92±0.17	0.61±0.35
21	21.34	2883.39	Alkene C29:1 (3)	5.52±0.58	3.02±1.74
22	21.43	2897.22	Alkene C29:1 (4)	7.93±0.96	1.06±0.61
23	21.58	2900.00	Nonacosane (C29)*	1.23±0.14	1.21±0.70
24	23.86	3065.26	Unknown 3	2.81±2.13	ND
25	24.05	3076.78	Alkene C31:1 (1)	0.95±1.76	0.08±0.5
26	24.19	3089.48	Alkene C31:1 (2)	1.01±0.12	0.60±0.35
27	24.35	3096.23	Alkene C31:1 (3)	1.92±0.21	1.14±0.66

ND = Not detected; * = Confirmed with synthetics; ** = Compared with the NIST library; RT = retention time; RI = retention index; Number in parenthesis = number of isomer.
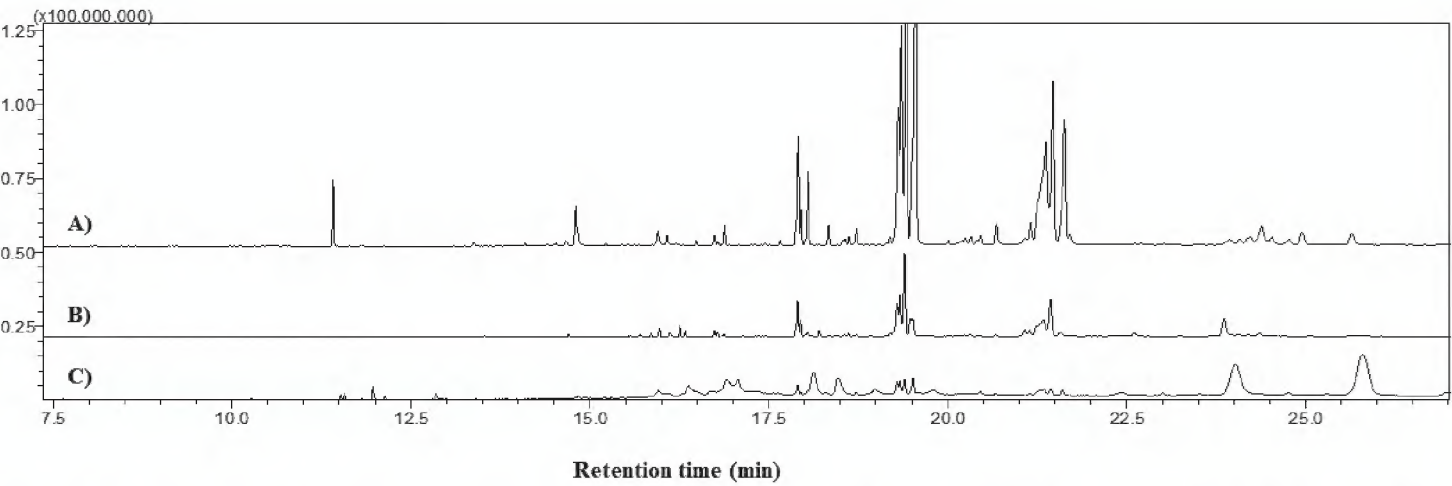
Figure 5. Chromatographic analysis of the extracts from *M. beecheii* **A** hexane wash of the Petri dish feeder **B** hexane extract of the labial gland **C** hexane extract of the mandibular gland.

Discussion

In this study, we demonstrated that *M. beecheii* foragers preferred to visit feeders that had been previously visited by their conspecifics over a clean feeder. This behavior has been observed in several species of the genus *Melipona*, such as *M. favosa* (Aguilar and Sommeijer 2001), *Melipona mandacaia* (Nieh et al. 2003), *Melipona seminigra* (Jarau et al. 2004b; Jarau et al. 2005), *M. panamica*, *M. scutellaris* (Nieh 1998; Roselino et al. 2016), and *M. solani* (Alavez-Rosas et al. 2017). Therefore, the main mechanism that stingless bees of the genus *Melipona* use is leaving chemical markers to guide their conspecifics to food sources, which could be considered as a clear proof that the chemicals are not signals with a specific meaning for the receiver but rather cues that have to be learned according to context specific meanings (Barth et al. 2008; Roselino et al. 2016). For example, it is known that *M. favosa* foragers constantly deposit anal excretions on food sources (Aguilar and Sommeijer 2001), while *M. mandacaia* foragers deposit anal droplets and a ventro-abdominal odor, an event not previously described (Nieh et al. 2003). *M. seminigra* foragers deposit a pheromone secreted by their claw retractor tendons on food sources (Jarau et al. 2004b; Jarau et al. 2005). *M. panamica* and *M. scutellaris* deposit and associate olfactory marks that influence the orientation of conspecifics to a source food (Nieh 1998; Roselino et al. 2016), and *M. solani* uses the secretion of its labial gland (Alavez-Rosas et al. 2017). Through behavioral experiments, we showed that *M. beecheii* foragers prefer to visit feeders that contain extracts of labial gland secretion and, moreover, exhibited antennal responses to these extracts. By washing the food container, we also demonstrated that foragers, indeed, deposit the labial gland secretion on food. The chemical composition of the extracts from the hexane wash corresponds to the chemical composition of the labial gland extract, suggesting that labial gland secretion may contribute to chemicals left behind by foraging bees at food sources. Regarding the chemical composition of the deposited marks, there is no information (Nieh 1998; Roselino et al. 2016), except for *M. solani*, whose foragers deposit a mixture of hydrocarbons and methyl oleate (Alavez-Rosas et al. 2017). In our study, we found that in the secretion from the *M. beecheii* labial gland the most abundant compounds were alkene C25:1 (1), alkene C27:1 (1), alkene C27:1 (2), alkene C27:1 (3), alkene C29:1 (4), and unknown 3. Heneicosene, methyl stearate, and tricosane are found in small amounts. The main composition of the labial gland includes hydrocarbon-type compounds; we believe that the function of this type of compounds is short-range recognition. In the field, *M. beecheii* foragers deposit their labial gland secretions to mark feeding sites and which might help to promote visits by their conspecifics; the presence of these compounds likely indicates to the bees that it is a food-rich resource, while flower volatiles attract the bees to the food source.

Interestingly, during the analysis of the labial gland extracts (data no shown) we found a group of samples with the same composition but in different proportions. According to the literature, *M. beecheii* bees first fly at the age of 33 days, probably to

orient themselves in the environment; the first foraging flight is at 40-days-old, although at 20 days of age, a constant proportion of bees leave the hive to feed (Biesmeijer and Tóth 1998; Medina-Medina et al. 2014), and thus it is concluded that age is not a factor for initiating the search for food. For this reason, we can infer that, although the profiles found here are not significant, there are foragers of different ages that search for food.

When the mandibular gland extract was evaluated, foragers behavior was more aggressive. Bee antennae responded to the labial and mandibular gland extracts similarly. The responses to the labial gland were stronger, while responses to the mandibular gland were weak, but stronger than the control. The *M. beecheii* mandibular gland possesses rose oxide isomers, which cause high levels of defense behavior, as do geraniol and farnesyl acetate that, when used at levels similar to those of the mandibular extract, cause more pronounced defense reactions than the rose oxide isomer (Cruz-López et al. 2005). These compounds were possibly detected by the worker bee antennae and induced a stronger response to the mandibular extract than to the control.

Conclusion

In sum, our results indicate that *M. beecheii* foragers prefer to visit feeders have been previously visited by their conspecifics. Labial gland secretion may contribute to chemicals left behind by foraging bees at food sources, and more sophisticated analyses are needed to come to a definite conclusion. The secretion found at feeding sites is composed of a mixture of unsaturated hydrocarbons, straight chain hydrocarbons, and small quantities of esters. The main components are a mixture of alkene C27:1 isomers. Further studies are needed to identify and synthetize if required the compounds used by *M. beecheii* foragers to recruit conspecifics toward the food resources.

Acknowledgments

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